Regular paper

Protective effects of cassia seed ethanol extract against carbon tetrachloride-induced liver injury in mice

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Oxidative stress has been recognized as a critical pathogenetic mechanism for the initiation and the progression of hepatic injury in a variety of liver disorders. Antioxidants, including many natural compounds or extracts, have been used to cope with liver disorders. The present study was designed to investigate the hepatoprotective effects of cassia seed ethanol extract (CSE) in carbon tetrachloride (CCl$_4$)-induced liver injury in mice. The animals were pre-treated with different doses of CSE (0.5, 1.0, 2.0 g/kg body weight) or distilled water for 5 days, then were injected intraperitoneally with CCl$_4$ (0.1% in corn oil, v/v, 20 ml/kg body weight), and sacrificed at 16 hours after CCl$_4$ exposure. The serum aminotransferase activities, histopathological changes, hepatic and mitochondrial antioxidant indexes, and cytochrome P450 2E1 (CYP2E1) activities were examined. Consistent with previous studies, acute CCl$_4$ administration caused great lesion to the liver, shown by the elevation of the serum aminotransferase activities, mitochondria membrane permeability transition (_MPT_), and the ballooning degeneration of hepatocytes. However, these adverse effects were all significantly inhibited by CSE pretreatment. CCl$_4$-induced decrease of the CYP2E1 activity was dose-dependently inhibited by CSE pretreatment. Furthermore, CSE dramatically decreased the hepatic and mitochondrial malondialdehyde (MDA) levels, increased the hepatic and mitochondrial glutathione (GSH) levels, and restored the activities of superoxide dismutase (SOD), glutathione reductase (GR), and glutathione S-transferase (GST). These results suggested that CSE could protect mice against CCl$_4$-induced liver injury _via_ enhancement of the antioxidant capacity.

Key words: hepatoprotective effects, antioxidant, cassia seed extract, carbon tetrachloride, CYP2E1

INTRODUCTION

Oxidative stress refers to a cell's state characterized by excessive production of reactive oxygen species (ROS) and/or reduction in antioxidant defense system (Franco _et al._, 2008). To resist the ROS, organisms possess a host of antioxidant defense systems, including non-enzymatic antioxidants, mainly glutathione (GSH), and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). However, oxidative stress still could occur due to an excess production of ROS and/or a defect of antioxidant molecules, which could cause damage to macromolecules such as DNA, proteins, and lipids. Accumulating evidence has demonstrated that oxidative stress plays a critical role in the initiation and progression of a variety of liver disorders (Medina & Moreno-Otero, 2005), and many natural antioxidants have been tried to prevent oxidative stress-mediated liver injury (Hewawasam _et al._, 2003; Nwozo & Oyinloye, 2011; Skinner & Rangasami, 2002; Srivastava & Shivanandappa, 2006). The main advantage of nature compounds or extracts is their mild action in comparison to chemically synthesized drugs.

The seeds of _Cassia obtusifolia_ (Leguminosae) are used as a traditional medicine in Japan, Korea and China for the treatment of eye inflammation, photophobia and lacrimation, dysentery, headache, as well as dizziness (Drever _et al._, 2008; Guo _et al._, 1998). The chemical constituents of the seeds have been investigated, and a number of anthraquinones isolated. Biological activities of the _Cassia obtusifolia_ extract (COE) including antibacterial activity, inhibitory effects on histamine release, antiplatelet aggregation, memory protection and neuroprotection have been reported (Drever _et al._, 2008; Kim _et al._, 2007; Kitanaka _et al._, 1998; Sung _et al._, 2004; Yun-Choi _et al._, 1990). Hepatoprotective effects of _Cassia obtusifolia_ have also been investigated in several studies in which Cassia leaves extracts showed hepatoprotective activity against paracetamol, ethyl alcohol and diethylnitrosamine (Bhakta _et al._, 2001; Jafri _et al._, 1999; Pradeep _et al._, 2007). However, whether the seeds of the plant possess hepatoprotective effects remains unclear.

Carbon tetrachloride (CCl$_4$) is a well-known model compound for producing chemical-induced hepatic injury, which has been widely used for the investigation of hepatoprotective agents (Hewawasam _et al._, 2003; Hsu _et al._, 2008; Lee _et al._, 2008). CCl$_4$ toxicity is initiated by the bioactivation of the CCl$_4$ molecule to trichloromethyl free radicals catalyzed by cytochrome P450 isoenzymes (especially cytochrome P4502E1, CYP2E1) (Comporti, 1989; Manibusan _et al._, 2007). The overproduced free radicals could overwhelm the antioxidant defense system of organisms, resulting in toxicity. CYPE21 plays a pivotal role in CCl$_4$-induced liver injury, and CYP2E1 (-/-) mice did not exhibit apparent liver injury after treatment with CCl$_4$ (Avasarala _et al._, 2006).

The current study was designed to investigate the hepatoprotective effects of Cassia seed extract (CSE) in mice exposed to CCl$_4$. The serum aminotransferase activities, histopathological changes, activities of CYP2E1, and changes of the hepatic and mitochondrial antioxi-

dant systems were measured for evaluation of the protective effects and exploration of the potential mechanisms.

MATERIALS AND METHODS

Materials. Commercial assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), GSH, SOD, GPx, and glutathione reductase (GR) were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Aniline and NADPH were purchased from Sigma (St. Louis, MO, USA). BCA protein assay kit was provided by Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals were of the highest quality commercially available.

Preparation of Cassia seed ethanol extract. Cassia seeds, bought from the local pharmacy, were mixed with 8 volumes of ethanol (80%, v/v) for 1 hour, and subjected to continuous hot extraction (60°C, 2 hours). The resulting extract was filtered and the residue was mixed with another 8 volumes of ethanol (80%, v/v) for further extraction (60°C, 2 hours). The combined ethanol extract was evaporated to dryness under reduced pressure to yield ethanol extract (11.0% of the mass of seeds) and stored at 4°C for use.

Animal treatments. Male Kun-Ming mice, weighing 18–22 g, were provided by the Laboratory Animal Center of Shandong University (Jinan, China). The mice had free access to commercial food and tap water, and were maintained in a temperature-controlled environment with a 12-h dark/light cycle. After 7 days of acclimation, the mice were randomized into 5 groups (n = 10). The mice were treated with CSE (0.5, 1.0, or 2.0 g/kg body weight) or distilled water (control group and CCl_4 group) by gavage for consecutive 5 days. Three hours after the last dosing, the mice in the CCl_4 and CSE groups were intraperitoneally injected with CCl_4 (0.1%, v/v, dissolved in corn oil, 20 ml/kg body weight) to induce acute liver injury, while the mice in the control group were administered with equal volume of corn oil. The animals were sacrificed at 16 hours after CCl_4 intoxication. Blood samples were collected for the measurement of serum aminotransferase activities, while the liver was quickly dissected. Portion of the liver was fixed in 10% formalin in 10 mM phosphate-buffered saline (PBS) for histological examination, while the remaining parts were snapped in liquid nitrogen and then stored at –80°C until analysis. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals.

Measurement of the ALT and AST activities. The blood samples were centrifuged (2500 rpm × 15 min, 4°C) for serum collection. The activities of serum ALT and AST were determined using an autoanalyzer (Shimadzu CL-7200, Japan).

Histopathological examination. Fresh liver was fixed in 10% formalin in 10 mM phosphate-buffered saline (PBS) (pH 7.4) for 18 h, and then were embedded in paraplast. Tissue sections (about 5 μm) were prepared, deparaffinized, dehydrated, stained with haematoxylin and eosin (H&E), and examined under light microscopy (Olympus, Japan).

Determination of hepatic and mitochondrial lipid peroxidation (LPO) and GSH levels. Liver tissue was homogenized in 9 volumes of cold buffer (0.01 M Tris/HCl, 0.0001 M EDTA-Na_2, 0.01 M saccharose, and 0.8% saline, pH 7.4) at 4°C. The homogenate was cen-

trifuged at 4°C (2500 rpm × 15 min) and the supernatant was used for the determination of the antioxidant system. MDA was used as a marker for the LPO assay. MDA and GSH levels were measured colorimetrically according to the instructions of commercial assay kits.

Mitochondria isolation and function assay. Mitochondria isolation and mitochondrial permeability transition (MPT) assay were performed as described previously (Song et al., 2003). Briefly, freshly prepared mitochondria (0.5 mg protein/ml) were equilibrated in 1 ml incubation buffer (containing 250 mM sucrose, 10 mM K-HEPES, 2 mM KH_2PO_4, 5 mM sodium succinate, pH 7.2) at 25°C for 10 min. Mitochondrial swelling was initiated by the addition of 0.3 mM calcium chloride. The decrease of the absorbance at 540 nm was determined in consecutive 5 min.

Determination of the activities of antioxidant enzymes. The activities of SOD, GR, GPx, and GST were measured using commercial assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer's instructions. SOD activity was measured following the reduction of nitrite by a xanthine–xanthine oxidase system which is a superoxide anion generator. GR activity was quantified by the depletion of NADPH measured at 340 nm. GPx and GST activities were assayed by the decrease of the GSH levels, which can be determined at 412 nm. The activities were expressed as U/mg protein, U/g protein, U/mg protein and U/mg protein, respectively.

Measurement of the CYP2E1 activity. Liver samples were homogenized in 4 volumes (w/v) of cold buffer containing 10 mM Tris-HCl, 0.15 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 0.01 mM phenylmethoxysulfonyl fluoride. Hepatic microsomal fractions were obtained by ultracentrifugation. Aniline hydroxylase (ANH) was determined as a specific biomarker for CYP2E1 activity with aniline as the substrate according to the procedure described previously (Pahan et al., 1997; Roberts et al., 1995; Yue et al., 2009; Zeng et al., 2009).

Statistical analysis. All data was expressed as mean ± S.D. SPSS 13.0 statistical software was used for statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. Differences were considered to be statistically significant at $P<0.05$ level.

RESULTS

CSE attenuates acute CCl_4-induced liver damage

The ALT and AST activities in mice in the CCl_4 group were significantly higher than those of the control mice ($P<0.01$). However, pretreatment with CSE markedly blunted these alterations in a dose-dependent manner (Fig. 1). Histological examination corresponded well with the biochemical analysis. The liver sections of CCl_4–intoxicated mice showed moderate ballooning degeneration and inflammatory cell infiltration, which were much milder in liver sections of the CSE-pretreated mice (Fig.2).

Effect of CSE on Ca^{2+}-induced mitochondrial permeability transition

MPT, characterized by the progressive permeabilization of the inner mitochondrial membrane, is a reliable marker for mitochondrial function. In order to investigate whether CSE could attenuate acute CCl_4-induced
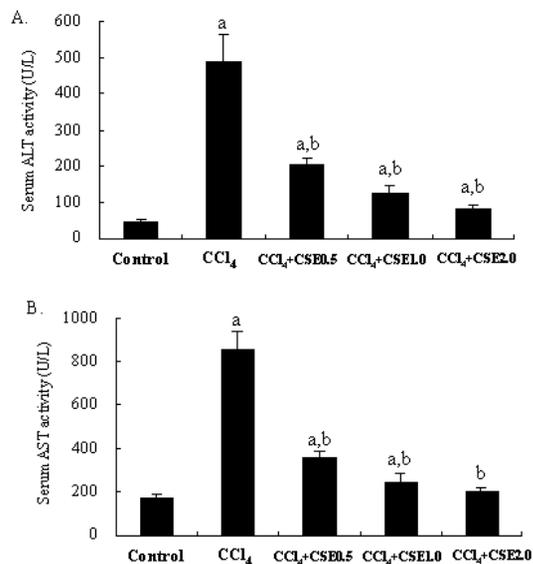
Figure 1. CSE inhibited CCl$_4$-induced increase of aminotransferase release.
Activities of serum ALT and AST were detected by an automatic analyzer using commercial assay kits, and the data were expressed as U/L (n=10). [a]$P<0.05$, compared with the control group; [b]$P<0.05$, compared with the CCl$_4$ group.
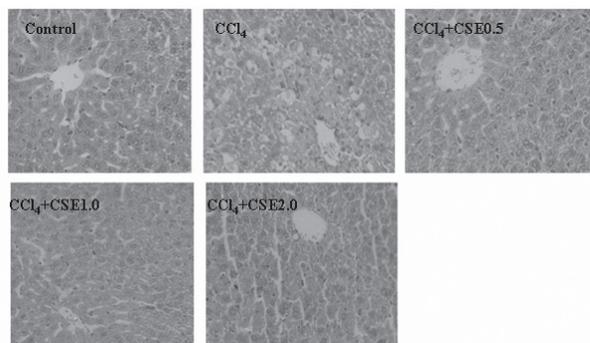

Figure 2. Effects of CSE on hepatic morphological changes in CCl$_4$-exposed mice.
Tissues sections (about 5 μm) were prepared, deparaffinized, dehydrated, stained with haematoxylin and eosin (H&E) by standard techniques. Magnification, 400×.
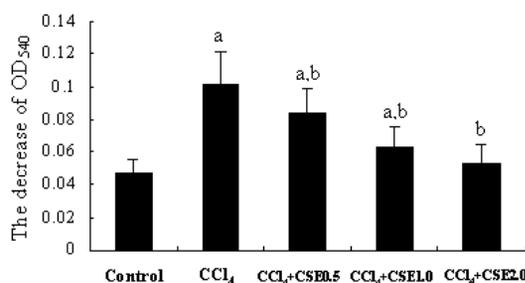

Figure 3. Effects of CSE and CCl$_4$ on Ca^{2+}-induced mitochondrial permeability transition.
Mitochondria were isolated by differential centrifugation, and diluted to 0.5 mg/ml in incubation buffer (250 mM sucrose, 10 mM K-HEPES, 2 mM KH$_2$PO$_4$, 5 mM sodium succinate, pH 7.2). Mitochondrial swelling was initiated by the addition of 0.3 mM calcium chloride. The decrease of absorbance at 540 nm was determined in consecutive 5 min. [a]$P<0.05$, compared with the control group; [b]$P<0.05$, compared with the CCl$_4$ group.

mitochondrial damage, we examined the effects of CSE and CCl$_4$ on Ca^{2+}-induced hepatic mitochondrial permeability transition. The data clearly showed that the absorbance at 540 nm (OD$_{540}$) was obviously decreased after the addition of Ca^{2+}. The mitochondria isolated from the CCl$_4$-group mice were more sensitive to Ca^{2+} as shown by a quick decline of OD$_{540}$. However, CSE attenuated Ca^{2+}-induced MPT, as shown by slow decline of OD$_{540}$ which mimicked that of control group mice (Fig. 3).

Effects of CSE on hepatic and mitochondrial MDA and GSH levels

Acute CCl$_4$ exposure resulted in the enhancement of LPO in the liver homogenate as well as in isolated mitochondria. Compared with those of the control group, the MDA levels of liver homogenate and mitochondria were increased by 88.03% ($P<0.01$) and 131.76% ($P<0.01$), respectively, which was dramatically inhibited by CSE treatment (Fig. 4A). The hepatic and mitochondrial GSH levels of the CCl$_4$-group mice were all markedly decreased compared to those of the control mice ($P<0.01$). These adverse effects were significantly attenuated in CSE-pretreated mice. Compared with those of CCl$_4$-group mice, the hepatic GSH levels in CSE-pretreated mice were increased by 47.77%, 55.11%, and 63.95% ($P<0.01$), respectively, while the mitochondrial GSH levels were increased by 92.43%, 107.19%, and 141.38% ($P<0.01$), respectively (Fig. 4B).

Effects of CSE on the activities of hepatic antioxidant enzymes

Compared with the control group, CCl$_4$ reduced the activities of SOD, GR and GST by 34.66%, 13.25% and 26.06% ($P<0.01$), respectively, while the GPx activity showed no significant changes ($P>0.05$). CSE dramatically boosted the activities of SOD, GR and GST ($P<0.05$), but it did not affect the GPx activity (Table 1).

Effect of CSE on CYP2E1 activity

In view of the critical role of CYP2E1 in the bioactivation of CCl$_4$, the effects of CSE on the enzyme were examined. CCl$_4$ caused a dramatic decrease of CYP2E1 activity, which was restored by CSE pretreatment (Fig. 5).

DISCUSSION

In the current study, the hepatoprotective effects of CSE were investigated in CCl$_4$-exposed mice, which is a widely used animal model. The well-known biomarkers of liver injury (ALT and AST), the mitochondrial function indexes (Ca^{2+}-induced MPT), and histological changes, were examined for the evaluation of the protective effects of CSE. Consistent with previous studies, our study confirmed that acute CCl$_4$ exposure damaged the liver, as shown by the elevation of the serum aminotransferase activities, the dysfunction of mitochondria, and morphological changes. Interestingly, these adverse effects were significantly attenuated by CSE pretreatment, which indicated a prominent hepatoprotective effect of CSE against CCl$_4$.

It has been well documented that CCl$_4$ toxicity results from the bioactivation of the CCl$_4$ molecule to trichloromethyl free radicals by cytochrome P450 isoenzymes (Comporti, 1989; Manibusan et al., 2007; Sheweita et al., 2001b). The free radicals are highly reactive and are capable of covalently binding locally to cellular macromolecules with preference for fatty acids from membrane
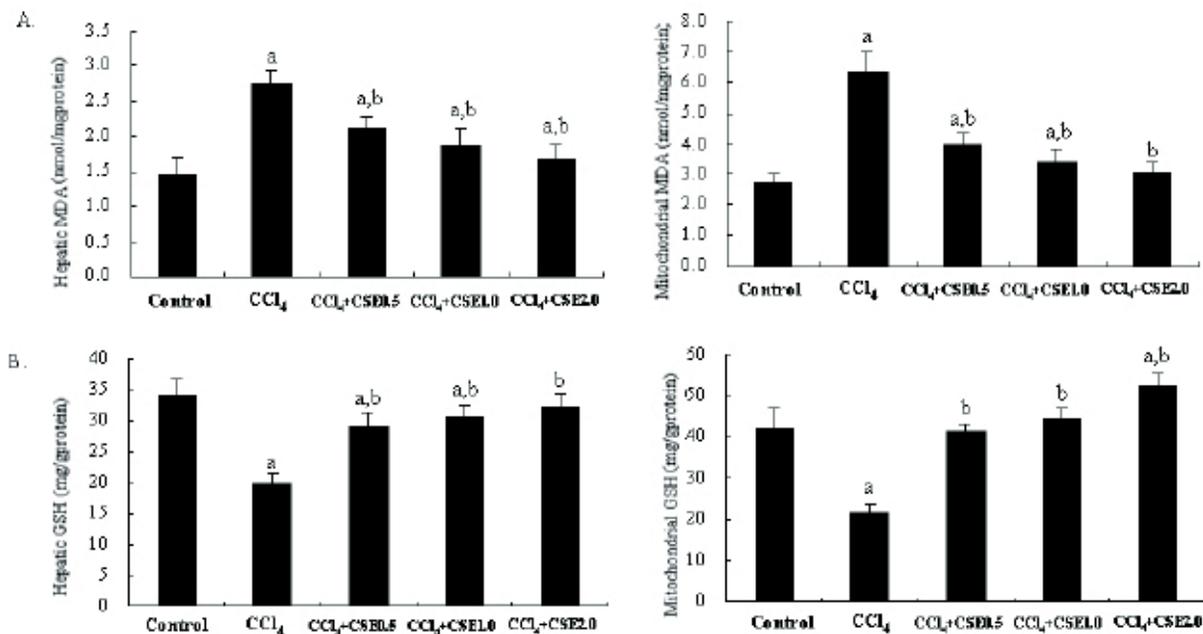
Figure 4. Effects of CSE and CCl₄ on hepatic and mitochondrial MDA and GSH levels.
Data are shown as mean ± S.D. (n=10). [a]$P<0.05$, compared with the control group; [b]$P<0.05$, compared with the CCl₄ group.

phospholipids. Free radicals and the following lipid per-oxidation can attack polyunsaturated fatty acids in the biomembrane, damage membrane enzymes, and disrupt cell energy generation and protein synthesis. Mitochondria contain large amounts of polyunsaturated fatty acids in phospholipids and thus are usually more susceptible to oxidative stress. In the present study, MDA level was used as a biomarker of lipid peroxidation, and we found that the hepatic and mitochondrial MDA levels were significantly increased in CCl₄ group by 88.03% and 137.16%, respectively, when compared to those of the control mice. Similarly to the changes of serum aminotransferase activities, the hepatic MDA levels of CSE-pretreated mice were significantly lower than in the CCl₄ group, which indicated that CSE pretreatment inhibited acute CCl₄-induced LPO (Fig. 4).

In order to resist the oxidative stress, organisms possess a host of antioxidant systems, including the non-enzymatic system (mainly GSH) and a series of antioxidant enzymes (e.g., SOD, GR, GPx and GST). Among them, the GSH-related antioxidant system plays crucial role in the antioxidant defense, nutrient metabolism, and regulation of cellular events (Wu et al., 2004). GSH is the predominant low-molecular-weight thiol in animal cells, and can effectively scavenge free radicals and other ROS (e.g., hydroxyl radical, lipid peroxy radical,

peroxynitrite, and H₂O₂) through non-enzymatic and enzymatic process in which the GSH is oxidized to glutathione disulfide (GSSG). The GSSG can be reduced to GSH by glutathione reductase (GR) with the consumption of NADPH (Fang et al., 2002). GPx catalyzes the GSH-dependent reduction of H₂O₂ and other peroxides. Moreover, GSH can also react with various electrophiles, physiological metabolites and xenobiotics to form mercapturates, a reaction catalyzed by GST (Lei, 2002). In addition to the GSH-related antioxidant system, SOD has also been demonstrated to play an important role in antioxidant defense system. Similarly to previous reports, acute CCl₄ exposure significantly decreased the activities of SOD, GR, GST and the levels of GSH, while the GPx activity was not affected (Di Simplicio & Mannervik, 1983; Dwivedi et al., 2006; Hsu et al., 2008; Sheweita et al., 2001a). However, CSE pretreatment effectively blocked the CCl₄-induced impairment of the antioxidant system (Table 1). CCl₄ dramatically decreased the hepatic GSH level (42.38%), and the mitochondrial GSH pool was impaired even more as expected (49.10%). Compared with those in mice of the CCl₄ group, the hepatic and mitochondrial GSH levels were simultaneously increased in mice of the CSE-pretreated groups in a dose-dependent manner. The activation of the SOD and GST could accelerate the clearance of free radicals, while the

Table 1. Effects of CSE on activities of hepatic antioxidant enzymes

Group	SOD (U/mg protein)	GR (U/g ptotein)	GPx (U/mg protein)	GST (U/mg protein)
Control	387.56±27.65	7.73±0.31	144.12±17.59	28.21±2.93
CCl₄	253.24±51.62[a]	6.71±0.40[a]	131.74±16.52	20.86±1.32[a]
CCl₄+ CSE 0.5	343.90±23.97[b]	7.23±0.21[b]	135.06±13.51	29.37±2.80[b]
CCl₄+ CSE 1.0	363.94±26.06[b]	7.78±0.29[b]	134.71±14.56	31.16±2.54[b]
CCl₄+ CSE 2.0	392.69±39.33[b]	8.13±0.31[b]	139.85±13.80	32.51±2.15[b]

Data are shown as mean ± S.D. (n = 10). [a]P<0.05, compared with the control group; [b]P<0.05, compared with the CCl₄ group.
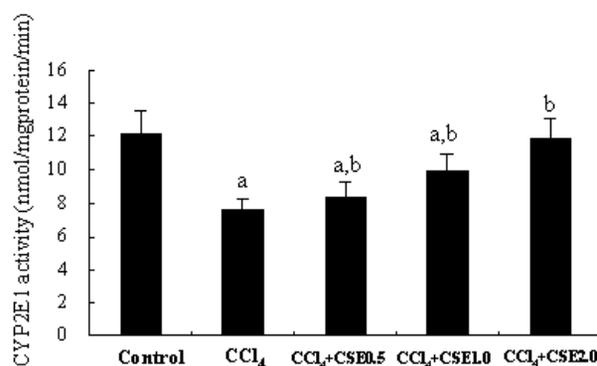
Figure 5. Effects of CSE and CCl₄ on CYP2E1 activity.
Liver microsome was prepared by ultracentrifugation. The activity of CYP2E1 was measured with aniline as substrate. Data are shown as mean ± S.D. [a]$P<0.05$, compared with the control group; [b]$P<0.05$, compared with the CCl₄ group.

activation of GR could hasten the biotransformation of GSSG to GSH. All these data suggest that the protective effects of CSE could be associated with the antioxidant activity.

CCl₄-induced toxicity requires CCl₄ metabolism by CYP2E1. In previous studies, CYP2E1 inhibitors protected against CCl₄-induced hepatotoxicity (Fukao et al., 2004; Yang et al., 2001). Furthermore, several studies showed that CCl₄ did not induce apparent liver damage in CYP2E1-null mice, which provides direct evidence for the important role of CYP2E1 in CCl₄-induced toxicity (Avasarala et al., 2006; Wong et al., 1998). In the current study, we found that CYP2E1 activity was significantly decreased by CCl₄ treatment, and was restored by CSE pretreatment (Fig. 5). Similar results have also been obtained in other studies (Tierney et al., 1992; Weerachayaphorn et al., 2010). In fact, previous studies have demonstrated that the reactive radicals produced from CCl₄ could bind covalently to CYP2E1, causing suicidal inactivation of the enzyme (Fernandez et al., 1982). The less pronounced reduction of the CYP2E1 activity in CSE-treated mice could also reflect the protection of CSE against CCl₄ toxicity.

In summary, the current study demonstrated that CSE showed a potent protective effect against CCl₄-induced liver injury. CSE pretreatment significantly inhibited the increase of the serum aminotransferase activities, attenuated oxidative stress-induced mitochondrial dysfunction, and decreased the pathological changes. CSE boosted the activities of antioxidant enzymes and elevated hepatic GSH levels, which could account for its hepatoprotective activity.

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